**TITLE:** Non-neutralizing antibodies against factor VIII and risk of inhibitor development in patients with severe hemophilia A.

**Short Title:** Anti-FVIII non-neutralizing antibodies and inhibitor.

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- Non-neutralizing antibodies against factor VIII are detected in untreated or minimally treated patients with hemophilia A.
- The presence of non-neutralizing antibodies is associated with a substantially increased risk of inhibitor development.

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Keywords: severe hemophilia A, inhibitor, non-neutralizing antibody, previously untreated patient.
ABSTRACT

The development of anti-factor VIII (FVIII) neutralizing antibodies (inhibitors) is the major complication in hemophilia A. Non-neutralizing antibodies (NNAs) have not only been detected in hemophilia patients but also in unaffected individuals. The aim of this study was to assess the prevalence of NNAs in a cohort of previously untreated or minimally treated patients with hemophilia A, and to evaluate whether their presence is associated with the development of inhibitors. Plasma samples of 237 patients with severe hemophilia A enrolled in the SIPPET trial were collected before any exposure to FVIII concentrates and analyzed for the presence of anti-FVIII NNAs. Patients were followed for the development of neutralizing antibodies. NNAs were found in 18/237 (7.6%) of patients at screening, with a clear age gradient. Of those with NNAs, seven patients subsequently developed an inhibitor, for a cumulative incidence of 45.4% (95% confidence interval (CI95) 19.5-71.3%), whereas among those without NNAs 64/219 (29%) developed an inhibitor (cumulative incidence 34.0%, CI95 27.1-40.9%). In Cox regression, patients with NNAs at screening had a 83% higher incidence of inhibitor development than patients without NNAs (hazard ratio (HR) 1.83, CI95 0.84-3.99). For high-titer inhibitors, the rate was almost 3-fold increased (HR 2.74, CI95 1.23-6.12). These associations did not materially change after adjustment. The presence of anti-FVIII NNAs in patients with severe hemophilia A not previously exposed to FVIII concentrates is associated with an increased incidence of inhibitors.
INTRODUCTION

The development of alloantibodies neutralizing factor VIII (FVIII) coagulant activity (inhibitors) represents the main complication of treatment of hemophilia A. It occurs in approximately one third of previously untreated patients (PUPs) and causes substantial morbidity, mortality and costs for the healthcare system. The causes for inhibitor development are not fully understood, but some risk factors have been identified. FVIII inhibitors consist of a polyclonal population of antibodies that are targeted to multiple antigenic sites within the A2, A3 and C2 domains of the protein. In addition to inhibitors, anti-FVIII antibodies are present in healthy individuals and patients affected with hemophilia A without exerting coagulant inhibitory activity. Several laboratory platforms for the detection of total FVIII binding antibodies have been developed, based upon immunoblotting assay, fluorescence immune assay and enzyme-linked immunosorbent assay (ELISA). With these assays non-neutralizing antibodies (NNAs) directed towards non-functional FVIII epitopes that escape detection by means of functional assays have been identified. Their prevalence is approximately 2-3% in healthy individuals, while estimates in hemophilia patients with different degrees of severity of disease vary widely, from 12% to 54%. Although NNAs and inhibitors are not distinguishable based on their isotypes, clonality and epitopes, recent data published by Hofbauer indicate that anti-FVIII IgG with inhibitory activity have an up to 100-fold higher affinity for FVIII than those without inhibitory activity. Based on studies on cross-reactivity it has been also suggested that FVIII inhibitors in hemophilia A patients originate from the expansion of a natural anti-FVIII clone of B lymphocytes that exists before any treatment with FVIII and secretes anti-FVIII antibodies similar to the natural antibodies found in healthy individuals. Furthermore,
FVIII inhibitors seem to be produced by a B cell clone that has undergone an antigen-driven affinity-maturation and hypermutation of the V-region\textsuperscript{33}. With this as background, what is not known pertaining to NNAs is their presence before any exposure to FVIII concentrates and their relationship with subsequent inhibitor development, even though Boylan et al.\textsuperscript{15} already suggested that NNAs could be an early sign of subsequent inhibitor development. Currently available data on NNAs stem from large but heterogeneous cohorts of multi-transfused patients, some with previous but then eradicated inhibitors, and others during immune tolerance induction for inhibitor eradication. Furthermore, the majority of studies were cross-sectional in design, and could not assess temporal relations. In particular, none of the previous studies was performed on plasma samples collected before any FVIII concentrate exposure\textsuperscript{11-31}.

The aim of this study was to determine the prevalence and significance of anti-FVIII NNAs in patients enrolled in the prospective randomized SIPPET study who were screened for neutralizing and non-neutralizing anti-FVIII antibodies before any treatment with FVIII concentrates.

**MATERIAL AND METHODS**

**Patients:**
This was a cohort study performed in the frame of SIPPET randomized trial\textsuperscript{35}. Patients aged <6 years, affected with severe hemophilia A, never exposed to FVIII concentrates, not or minimally exposed (less than 5 EDs) to blood components (whole blood, fresh frozen plasma, packed red cells, platelets or cryoprecipitate) and inhibitor negative by modified Bethesda assay were included and then randomized to treatment with a single plasma-derived (pdFVIII) or recombinant (rFVIII) FVIII concentrate. Patients were followed and monitored
at scheduled time-points for FVIII inhibitor occurrence. Data were collected on family history of hemophilia and inhibitor, age at screening, country site, FVIII gene mutations and FVIII sources (for more details, see reference); FVIII antigen levels (measured at screening by Asserachrom VIII:C Ag, Diagnostica Stago, Asnières sur Seine, France) were categorized as <1% and ≥ 1%. Minimally treated patients (MTPs) were defined as those exposed to less than 5 EDs with blood components before screening, whereas previously untreated patients (PUPs) were those never exposed to blood components. Screening was defined as the time of inclusion in the SIPPET trial, before any exposure to FVIII concentrate. At this time a blood sample was collected and patients were screened both for the presence of inhibitory antibodies using the Bethesda assay with the Nijmegen modification and stored for future determination of the presence of NNAs using an ELISA assay. Plasma samples collected at screening were available for 237 patients and were tested for anti-FVIII NNAs. Approval was obtained from medical ethics committee at each study center. Parents or guardians of all children provided written informed consent.

**Inhibitor testing**

Inhibitor testing at screening and follow-up was performed centrally at the Angelo Bianchi Bonomi Hemophilia and Thrombosis Centre, Milan, Italy, using the Bethesda assay with the Nijmegen modification. In case of inhibitor occurrence during follow-up, inhibitor levels were confirmed on a second sample within 14 days after the first positivity, and patients were followed for six months to establish whether the inhibitor was transient or persistent.

**Non-neutralizing antibody testing**

ELISA plates (96-wells NUNC Maxisorp, St. Louis, MO) were coated overnight at 4°C with 1.2 ug/mL of the full length rFVIII product Advate® (Baxter Healthcare Corporation,
Westlake Village, CA) previously dialyzed against phosphate buffered saline (PBS). The plates were then blocked for 2 h at room temperature with PBS containing 5% skim milk powder (Merck, Kenilworth, NJ). After blocking, the plates were washed 3 times with PBS 0.1% Tween-20 (Sigma, Merck, Kenilworth, NJ). The same washing procedure was performed between the different incubation steps. Samples were diluted in PBS, 1% skim milk powder (Merck, Kenilworth, NJ), 0.01% Tween-20 (Sigma, Merck, Kenilworth, NJ), added to the coated plates and incubated for 2 h at room temperature. After a washing step, the plates were incubated 1 h at room temperature with horseradish peroxidase (HRP)-anti human IgG (GE Healthcare, Little Chalfont, UK) diluted 1/2000 in PBS, 1% skim milk powder (Merck, Kenilworth, NJ), 0.01% Tween-20. O-phenylenediamine dihydrochloride (OPD) substrate (Sigma, Merck, Kenilworth, NJ) was added and the reaction stopped by adding 3 mol/L H₂SO₄. Absorbance was read at 492 and corrected for background at 620 nm. Each plasma sample was analyzed twice in different assay runs. In case of discrepancy with values deviating from the range of interassay variability (CV = 25%), a third assay was performed. The mean of two assay results or of the two closest values in the case of three assays was used. In the first run all plasma samples were diluted 1/10 and, in the second, samples with high IgG concentration were further diluted to have an absorbance value that fell within the linear portion of the standard curve. In each assay, a positive control sample (plasma with a high-titer inhibitor) diluted 1/40 and a negative control sample (normal pool plasma) diluted 1/10 were also used.

**Standard curve:** Total IgG from a plasma of a patient with hemophilia A and a high-titer inhibitor (600 BU) were purified on a Protein G-Sepharose (GE Healthcare, Little Chalfont, UK) column. Specific anti-FVIII IgG were then isolated by using affinity chromatography on Affi-Gel 10 (Bio-Rad, Hercules, CA) coupled with rFVIII (Advate®), 4000 IU/2mL of slurry gel. The affinity purified anti-FVIII specific IgGs were checked for purity by means of
sodium dodecyl sulphate – poly acrylamide gel electrophoresis (SDS PAGE electrophoresis) and used in the ELISA assay to construct the standard curve. The cut-off for positive anti-FVIII NNA was set at 1.64 µg/mL specific anti-FVIII IgG, corresponding to 100% specificity and 96% of sensitivity in the ROC curve constructed with the results of anti-FVIII IgG measured in 107 healthy individuals and 101 hemophilia A patients with positive FVIII inhibitor detected by Bethesda assay (mean 103.9 BU, median 14.5 BU, range: 0.5-1400 BU).

Statistical analysis

Prevalences were estimated as proportions with confidence intervals obtained by the exact binomial method of Clopper-Pearson. We used odds ratio (OR) and 95% confidence intervals (CI95) to assess putative determinants of NNA. Analysis by age at screening was done by stratifying patients in 5 age categories. Independent samples t-test was used to compare age between the two groups.

Kaplan-Meier survival analyses were performed to assess the cumulative incidence for both all inhibitors and high-titer inhibitors by NNAs serotype, and the incidence rates were compared with Cox regression survival analyses taking into account as covariates FVIII gene mutations (categorized as null- vs non-null mutation)\textsuperscript{35}, FVIII antigen levels, self-reported family history for inhibitor, trial treatment arm (pdFVIII or rFVIII), age at screening (in months) and country site. Adjustments in multivariate Cox models were each made individually, as there were too few events to include all variables in a single multivariate model. Confidence intervals were derived from this model. Due to the occurrence of two deaths during the trial\textsuperscript{35}, we performed a sensitivity analysis assuming that both patients had developed a high-titer inhibitor at the truncated follow-up instead of dying. Statistical analyses were performed within SPSS, version 23.0 (IBM Corp., Armonk, NY).
RESULTS

Characteristics of the study cohort

The mean age at screening was 18.3 months (median 13 months; range: 0-67 months). FVIII gene causative mutations were detected in 94.1% of the cases (n=223): 185 of them (83%) were carrying a null-mutation (large deletions, nonsense mutations, inversions and frameshift). In 107 of 237 patients (45.1%) there was a positive history of hemophilia in relatives, and 23 (21.5%) reported a positive family history of inhibitor. One hundred and twenty-one patients (51.1%) were randomized to treatment with pdFVIII and 116 (48.9%) to rFVIII. Of 237 patients followed for a mean of 27 ED (median 23 days; range: 1-50 days), 71 developed a FVIII inhibitor (30.0%), which in 48 (67.6%) was at high-titer.

Determinants of non-neutralizing antibodies towards FVIII

NNAs were detected at screening in 18/237 patients (7.6%; CI 4.9-11.7%). Table 1 shows the general characteristics of NNA-positive and NNA-negative subjects. Patients with NNAs were older than those without (mean 27.4 months; median 24 months; range: 2-59 months vs mean 17.5 months; median 12 months; range 0-67 months; p-value: 0.007), and NNA prevalence clearly increased with age (Table 2). Non-null mutations, measurable FVIII antigen levels in plasma and a positive family history of inhibitor were also associated with a higher prevalence of NNAs at screening (Table 2). Previous exposure to blood components was associated with a reduced prevalence (OR 0.49, CI 0.17-1.43). For all these associations, confidence intervals were wide due to the limited sample size, and none changed after adjustment for age.
NNAs and inhibitor development

Among patients positive for NNAs at screening (n = 18), seven subsequently developed an inhibitor, for a cumulative incidence of 45.4% (CI95 19.5-71.3%), whereas among those negative (n = 219) 64 developed an inhibitor, for a cumulative incidence of 34.0% (CI95 27.1-40.9%). In the NNA-positive group, all inhibitors were high-titer, whereas in the NNA-negative group, 41 of 64 inhibitors were high-titer, for a cumulative incidence of 21.9% (CI95 15.8-28.0%). Importantly, no inhibitor among NNA-positive subjects was transient, compared with 26.6% (17 out of 64 patients) of inhibitors in the NNA-negative group which were transient and disappeared within 6 months.

Figure 1 shows the Kaplan-Meier plots for all and high-titer inhibitors by the presence or absence of NNAs at screening. In univariate Cox regression, presence of NNAs was associated with an 83% higher incidence of inhibitors than in its absence (hazard ratio (HR) 1.83, CI95 0.84-3.99). For high-titer inhibitors the rate was almost three-fold increased (HR 2.74, CI95 1.23-6.12). In adjusted models, the effect of NNAs on inhibitor development if anything became stronger (Table 3).

Due to the stringency of our cut-off value for non-neutralizing anti-FVIII antibody positivity we also analyzed data using a lower cut-off of 1.035 µg/mL, corresponding to the highest value of the Youden index obtained from the ROC curve (99% of sensitivity, 98% of specificity) and results did not change: all inhibitors by NNA positivity HR 1.69 (CI95%: 0.97-2.96) and high-titer inhibitors HR 2.33 (CI95% 1.25-4.34). Two deaths occurred during the trial, both among NNA-negative patients. We performed a sensitivity analysis assuming that both patients had developed a high-titer inhibitor at the truncated follow-up instead of dying. We recalculated the crude hazard ratio by the presence of NNAs and the results did not change. The hazard ratio by NNAs presence was 1.78 (0.81-3.87) for all inhibitors, and 2.62 (1.18-5.84) for high-titer inhibitors. Severe non-fatal adverse events were nine episodes
of intracranial bleeding and two episodes of gastro-intestinal bleeds, all of them occurred in the NNA-negative group. Per protocol these adverse events were not considered a reason for withdrawal of the study, and subjects continued to be treated and completed the study. Further sensitivity analyses were performed for patients who were censored before reaching the predefined end-point of three years follow-up or 50 EDs, and no major deviations from the overall findings were found.

DISCUSSION

We studied a cohort of 237 patients with severe hemophilia A, previously untreated and minimally exposed to blood components and thereby at risk for FVIII inhibitor development after exposure to FVIII concentrates. A sensitive ELISA assay was used to detect NNAs at screening before any exposure to FVIII, which were present in 7.6% of patients. Age, non-null mutation, FVIII antigen levels and family history of inhibitor seemed to influence NNAs prevalence. Importantly, the presence of NNAs was associated with an increased risk of inhibitor development, particularly for high-titer inhibitors, with an nearly three-fold increased incidence rate.

In previously reports, NNA prevalence ranged from 2% to 3% in healthy people\textsuperscript{16,18,21,24} and from 12.2% to 53.8% in patients with hemophilia A\textsuperscript{13-22,24-25,29-30}. Several factors, particularly that all previous studies predominantly included multi-transfused patients, explain this large variation and the gaps of knowledge on their potential clinical role. For instance, the intron 22 inversion of FVIII mutation was associated with an increased frequency of NNAs in multi-transfused patients in one study\textsuperscript{29}, which was not confirmed in another\textsuperscript{20}. Among multi-transfused patients those with NNA were older than those with no NNAs in one study\textsuperscript{20}, but not in others\textsuperscript{13,30}. 
Given the increasing prevalence with age projected at 20% at an age of more than 40 months, NNAs can be considered an age-related phenomenon in patients previously untreated and minimally exposed to blood components, which may reflect maturation of the immune system and in particular of the adaptive immune system in growing children\textsuperscript{37}. This finding is also supported by the increase of natural IgGs during the first years to remain stable with aging\textsuperscript{38}.

We found a higher prevalence of NNAs at screening in untreated patients with non-null mutations than those with null mutations. One may hypothesize that patients with minor gene variations have residual non-functional plasma levels of FVIII antigen, which elicit an immune response before exposure to concentrates. This view is supported by the association between detectable FVIII antigen and NNAs. Measurable FVIII antigen in plasma\textsuperscript{39}, potential contamination with maternal blood during delivery\textsuperscript{40}, and the presence of milk fat globule epidermal growth factor-FVIII (MFGEGF-F8) (a glycoprotein with a strong homology with circulating FVIII in breast milk) might all be examples of early exposure\textsuperscript{41}. Possibly, the presence of NNAs before any specific treatment may be the expression of a natural anti-FVIII B cell clone, that exists independently to any environmental FVIII exposure\textsuperscript{42}.

Whereas some findings, particular those on determinants of NNAs had considerable statistical uncertainty and should be confirmed in a larger cohort, the association of NNAs at screening with subsequent inhibitor development was robust.

In our study, the presence of NNAs at screening in children not previously treated with any FVIII concentrate was associated with an almost 3-fold increased risk of development of high-titer inhibitors. In auto-immune diseases, such as systemic lupus erythematosus or rheumatoid arthritis, the appearance of autoantibodies often precedes the clinical onset of disease\textsuperscript{43-45}. A hypothetical explanation is that the autoantibody response needs to mature before acquiring such distinct characteristics as specificity of antigen recognition, or an
increase or shift in antigen recognition. Similarly, we found that detectable inhibitors, and mostly high-titer inhibitors, can be preceded by NNAs.

Furthermore, the effect of NNA on inhibitor development did not change its direction when adjusted for other variables, but the hazard ratio increased after adjustment for mutation. This can be explained by the inverse relations of null mutation with NNA presence on the one hand and inhibitor development on the other.

This study has some limitations. The follow-up of patients started from screening and not from birth, and therefore patients who died early were missed. Given the low fatality rate of hemophilia, and the implausibility of a differential death rate by NNAs, this is an unlikely source of bias.

In conclusion, the development of anti-FVIII inhibitors is a multicausal event and although several factors have been identified, none of them suffice to reliably predict the risk for an individual patient. The identification of NNAs as an additional marker for inhibitor development needs to be validated in further studies and once confirmed it could be useful to measure NNA immediately at diagnosis or before any exposure to FVIII products. This may contribute to improve prediction scores, which in turn may lead to individualized interventions tailored to reduce the risk of inhibitor formation.

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Authors’ contributions. A.C., F.R.R. and F.P. designed the study, interpreted data and wrote the manuscript. C.V. developed and validated ELISA assay, and performed the laboratory workout. I.G. collected data and did the literature search. R.P. performed data analysis and contributed in writing the manuscript. P.M.M. critically revised the manuscript. All authors
revised critically the work providing substantial input and gave final approval of the version to be published.

**Disclosure of interests.** R.P. has received travel support from Pfizer. P.M.M. reports honoraria for participating at advisory boards or as speaker at satellite symposia and educational meetings organized by Baxalta, Bayer, Grifols, Kedrion Biopharma, Novo Nordisk, Biotest and LFB. FP has received honoraria for participating as a speaker at satellite symposia and educational meetings organized by Bayer, Biotest, CSL Behring, Grifols, Novo Nordisk, and Sobi and she has received consulting fees from Kedrion Biopharma, LFB and Octapharma. She is recipient of research grant funding from Alexion, Biotest, Kedrion Biopharma, and Novo Nordisk received by Fondazione Luigi Villa. She is member of the Ablynx scientific advisory board.

The other authors have no conflicts to declare.

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REFERENCES:


Table 1: General characteristics and prevalence of non-neutralizing antibodies (NNAs) in the study cohort. Crude and age-adjusted odds ratio for the presence of NNAs according to potential determinants.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NNA negative</th>
<th>NNA positive</th>
<th>Crude odds ratio (CI 95%)</th>
<th>p-value</th>
<th>Age-adjusted odds ratio (CI 95%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at screening (months)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>17.5</td>
<td>27.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>12</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>67</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVIII mutation - n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>null-mutation</td>
<td>173 (93.5)</td>
<td>12 (6.5)</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td>1.0</td>
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<tr>
<td>non-null mutation</td>
<td>33 (86.8)</td>
<td>5 (13.2)</td>
<td>2.18 (0.72-6.61)</td>
<td>0.167</td>
<td>2.29 (0.74-7.10)</td>
<td>0.153</td>
</tr>
<tr>
<td>FVIII antigen levels</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt; 1%</td>
<td>195 (92.9)</td>
<td>15 (7.1)</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>≥ 1%</td>
<td>21 (87.5)</td>
<td>3 (12.5)</td>
<td>1.86 (0.50-6.94)</td>
<td>0.358</td>
<td>1.71 (0.45-6.50)</td>
<td>0.434</td>
</tr>
<tr>
<td>Family history of inhibitors - n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>69 (95.8)</td>
<td>3 (4.2)</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td>1.0</td>
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<tr>
<td>Yes</td>
<td>21 (91.3)</td>
<td>2 (8.7)</td>
<td>2.19 (0.34-14.00)</td>
<td>0.407</td>
<td>2.93 (0.41-20.95)</td>
<td>0.284</td>
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<tr>
<td>Previous exposure to blood components (&lt;5) - n (%)</td>
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<td></td>
<td></td>
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<tr>
<td>No</td>
<td>123 (90.4)</td>
<td>13 (9.6)</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td>1.0</td>
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<tr>
<td>Yes</td>
<td>96 (95.0)</td>
<td>5 (5.0)</td>
<td>0.49 (0.17-1.43)</td>
<td>0.193</td>
<td>0.41 (0.14-1.22)</td>
<td>0.110</td>
</tr>
</tbody>
</table>
Table 2: Distribution of NNAs over each age category and the corresponding risk estimates for the presence of NNAs.

<table>
<thead>
<tr>
<th>Age at screening (months)</th>
<th>Total</th>
<th>NNA-negative patients</th>
<th>NNA-positive patients</th>
<th>Odds ratio (CI95)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>237</td>
<td>219 (92.4)</td>
<td>18 (7.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 40</td>
<td>25</td>
<td>20 (80.0)</td>
<td>5 (20.0)</td>
<td>6.50 (1.43-29.52)</td>
<td>0.018</td>
</tr>
<tr>
<td>30 - 39</td>
<td>23</td>
<td>20 (87.0)</td>
<td>3 (13.0)</td>
<td>3.90 (0.73-20.80)</td>
<td>0.101</td>
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<tr>
<td>20 - 29</td>
<td>41</td>
<td>38 (92.7)</td>
<td>3 (7.3)</td>
<td>2.05 (0.40-10.65)</td>
<td>0.319</td>
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<td>10-19</td>
<td>67</td>
<td>63 (94.0)</td>
<td>4 (6.0)</td>
<td>1.65 (0.36-7.65)</td>
<td>0.331</td>
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<td>0 - 9</td>
<td>81</td>
<td>78 (96.3)</td>
<td>3 (3.7)</td>
<td>1.0 ref.</td>
<td></td>
</tr>
</tbody>
</table>

* p-value is the comparison of each age-category to the reference
Table 3. Cox regression for inhibitor development by NNA presence. Hazard ratio compares the risk of inhibitor development among those with NNA at screening versus those without. Adjustments in multivariate Cox models were each performed individually.

<table>
<thead>
<tr>
<th>Adjustment variable</th>
<th>all inhibitor</th>
<th>High-titer inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude*</td>
<td>1.83 (0.84-3.99)</td>
<td>2.74 (1.23-6.12)</td>
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<tr>
<td>Age at screening</td>
<td>1.91 (0.85-4.26)</td>
<td>2.75 (1.20-6.32)</td>
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<tr>
<td>Mutation</td>
<td>2.33 (1.05-5.15)</td>
<td>3.53 (1.56-8.00)</td>
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<tr>
<td>FVIII antigen level</td>
<td>1.73 (0.79-3.78)</td>
<td>2.95 (1.32-6.60)</td>
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<tr>
<td>Countries</td>
<td>2.36 (1.03-5.42)</td>
<td>3.91 (1.62-9.44)</td>
</tr>
<tr>
<td>Familiar history of inhibitor</td>
<td>1.96 (0.89-4.29)</td>
<td>3.03 (1.35-6.81)</td>
</tr>
<tr>
<td>Arm of treatment</td>
<td>1.73 (0.79-3.78)</td>
<td>2.61 (1.17-5.84)</td>
</tr>
<tr>
<td>Exposure to blood components</td>
<td>1.75 (0.80-3.86)</td>
<td>2.75 (1.22-6.22)</td>
</tr>
<tr>
<td>Mutation, countries</td>
<td>3.62 (1.49-9.42)</td>
<td>5.76 (2.17-15.26)</td>
</tr>
</tbody>
</table>

*un-adjusted model
Figures legend

**Figure 1:** Kaplan-Meier survival curves for inhibitor development by NNA presence. In figure 1A cumulative incidence for all inhibitor is plotted; in figure 1B cumulative incidence for high-titer inhibitor is plotted.
Non-neutralizing antibodies against factor VIII and risk of inhibitor development in patients with severe hemophilia A

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